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RESEARCH ARTICLE

Specific nutrient supplementation of defined serum-free medium for the improvement of CHO cells growth and t-PA production

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Abbreviations: GC: glucose conversion
LA: lactic acid
$Y_{p/s}$: yield

Recombinant CHO TF70R cells are able to grow and produce t-PA on serum-free medium BIOPRO1 (BioWhitaker Europe, Belgium). The purpose of the present study was to determine the effect of medium supplementation with vitamins, lipids, and specific amino acids on cell growth, t-PA production and biological functionality. Among vitamins, only biotin, folic acid, cobalamine and benzoic acid were required for improving growth and t-PA production. Lipid supplement allowed a significant increase cell concentration and t-PA specific activity and concentration, though its specific production rate decreased slightly. Medium supplementation with proline, serine and asparagine had also positive effects on cell growth. Besides, the addition of asparagine (even in the presence of glutamine) was essential for the production and biological quality of the t-PA. This systematic approach for media supplementation produced an increase in cell concentration around 100% and in t-PA production around 80%, with no detrimental effect on its biological activity. The effect of asparagine on t-PA production was unexpected and needs to be further studied. The above modifications of the production medium did not produce a significant

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effect on the metabolism of the main carbon and energy sources (glucose and glutamine) and the level of by-product formation (lactate and ammonia).

The use of animal cells, and in particular CHO (Chinese hamster ovary) cell lines, has gained great relevance for the production of recombinant proteins used in human therapy, such as growth factors, antithrombolitic and monoclonal antibodies. In fact, the CHO cell lines are widely used for being highly stable expression systems for heterologous genes (Wurm, 1997; Wurm, 2004), and for its relatively simple adaptation to adherence-independent growth in serum free media (Xie et al. 2003). Mammalian cell cultivation i*n vitro* requires a complex combination of nutrients, considering glucose and glutamine as main carbon, energy and nitrogen sources. Mineral salts, amino acids and vitamins are also required, while other essential nutrients, like growth factors, hormones, and receptor and transport proteins are required in small quantities as well. These latter compounds are usually provided by fetal bovine serum (FBS). Several authors have studied the relevance of amino acid supplementation in mammalian cell cultivation (Franek and Srámková, 1995; DiStefano et al. 1996; Stoll et al. 1996). Vitamins are required as cofactors in several reactions related to amino acid metabolism and protein functionality. Because of its complexity and high cost, optimization of media formulation is a key aspect for bioprocess development in animal cell cultivation. Suppression of FBS is nowadays enforced because of the well-reported drawbacks of its use (Werner, 2004), which precludes the approval of its use for the production of therapeutics (FDA, 1997; Derouazi et al. 2004). Besides, its considerable protein load severely hampers the purification of the product and its high cost can have a negative impact on the economy of the process. However, serum-free media implies a case to case design based on the particular supplements required for each strain. Chemically defined serum-free media have been extensively studied and several reviews have been published on the subject (Keen and Rapson, 1995; Lao and Shalla, 1996; Kim et al. 1999). Hormones and growth factors requirements are specific for each type of cell (Freshney, 2000; Xie et al. 2003). Albumin preparations, which are sometimes added instead of FBS, have proven to be effective for cell cultivation; its function usually is related to acting as a carrier for fatty acids and lipids and providing cells with protection from shear damage (Freshney, 2000). Being so, the direct addition of fatty acids and lipids could be a better option to formulate a defined medium with low protein content (Castro et al. 1995). Recombinant CHO TF 70R cells producing t-PA, as those used in this work, have been cultivated in DMEM: Ham's F12 50:50 medium with and without serum addition, showing that cell growth was somewhat lower in the serum free medium (Büntemeyer et al. 1987; Hansen and Emborg, 1994). This work refers to the improvement of the serum-free, low protein BIOPRO1 culture medium (BioWhitaker Europe, Belgium), by the addition of vitamins, cholesterol, fatty acids and specific amino acids, with the purpose of increasing cell concentration and t-PA production.

MATERIALS AND METHODS

Cell Culture

The cell line CHO TF 70R, kindly provided by Pharmacia and Upjohn (Stockholm, Sweden) has been manipulated genetically to produce t-PA. The basal medium used was a proprietary serum-free and low protein medium denominated BIOPRO1, kindly provided by BioWhitaker Europe (Verviers, Belgium). BIOPRO1 was supplemented with vitamins, chemical defined lipids concentrate (DLC) containing fatty acids and cholesterol (11905, GibcoBRL, USA), proline, serine and aspartic acid (Sigma, USA) as indicated in Table 1 for each culture (Ci). The medium was also supplemented with 20 mM of glucose 6 mM of glutamine in the different experiments. A control culture (CC) was also performed for each of the three series of experiments (Table 1). CC was performed to consider eventual variations associated to the physiological condition of the inoculum cells. As usual in animal cell cultivation, cells from the working cell bank are maintained from 4 to 6 weeks by periodical passages. A series of experiments is conducted with inoculum cells coming from the same number of passages, but this number can vary from one series of experiments to the other. The cultures were inoculated with cells from the mid-exponential phase of growth at a cell concentration of 0.18×10^6 to 0.20×10^6 cells ml^{-1}. The cell cultures were carried out in spinner flasks (Techne™, USA) with a working volume of 125 ml and stirred at 50 rpm in a CO_2 incubator (Forma Scientific CO_2 incubator, USA), at 37.0ºC, with 96% relative humidity in an atmosphere of 5% CO_2 in air.

Table 1. Medium culture composition on the different experiments.

	S1		S2				S3	
	C1	C2	C3	C4	C5	C6	C7	C8
V- TM	3X							
V-3BF		3X	3X	3X	6X	6X	3X	3X
DLC*			1	1.5	1	1.5	1.5	1.5
Ser + Pro							2X	2X
Asn								2X

*: 1.0 or 1.5 mL of DLC/100 mL BIOPRO.

Analysis

Cells were counted using a haemacytometer (Neubauer improved, Brand). Cell viability was determined by the trypan blue (Sigma, USA) exclusion method (1:1 mixture of 0.2% trypan blue in normal saline and cell sample). These analyses were done in quadruplicate with an error below 5 %. After cell counting, the remainder of each sample was centrifuged (5000 g, 1 min) to remove the cells

and the supernatant was frozen for further analysis. The following analyses were all done in duplicate, because they are quite reproducible with errors below 3%. Glucose and lactate concentrations were determined with an automated glucose and L-lactate Biochemical Analyzer (YSI 2700, Yellow Spring Instruments, USA). Ammonium concentration was determined by a flow injection analysis system as previously described (Campmajó et al. 1994). Amino acids were measured by HPLC (Waters 2695, USA), using a reversed phase column (AccQ·Tag Column, 3.9 mm x 150 mm, Waters, USA) and AccQ·Fluor reagent Kit (Waters, USA). t-PA was quantified by enzyme immunoassay (Imulyse t-PA, Biopool, USA). t-PA amidolytic activity was determined by using the synthetic chromogenic substrate S-2288 (Chromogenix, Italy). One international unit (UI) was defined as the amount of t-PA that catalyzes the hydrolysis of 1 µmol of substrate per minute at 37ºC, 0.1 M Tris buffer pH 8.4, containing 1.7 mM of S-2288.

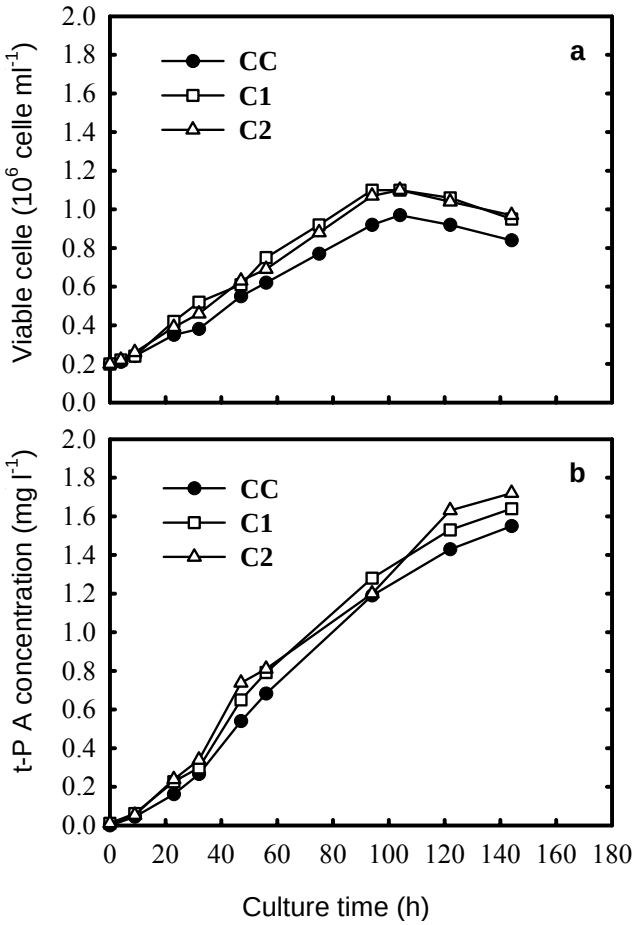

Figure 1. Effect of vitamins supplementation on CHO cells growth (a) and t-PA concentration (b). Results of the batch cultures using BIOPRO 1 (CC), BIOPRO 1 plus 3X V-TM (C1) and BIOPRO 1 plus 3X V-FB (C2).

Estimation of specific rates

Specific cell growth rate (µ), specific glucose and glutamine consumption rates, and specific lactate and t-PA production rates were estimated by plotting total cell concentration, cumulative glucose, and glutamine consumption, lactate and t-PA production, versus the integral of viable cells (IVC) and fitting the plots with a regression coefficient of close to one. The slope of this line was used as an average specific rate (Luan et al. 1987).

RESULTS AND DISCUSSION

BIOPRO 1 supplementation with vitamins was tested in the first series of cultures (S1). A vitamin concentrate, labeled as V-TM (M-6895, Sigma, USA), was added in culture C1 (8 mL of V-TM/100 mL BIOPRO1) so to produce an approximately threefold increase in all vitamins in BIOPRO 1. In C2 only the concentration of folic acid, cobalamine, biotin and benzoic was increased threefold by adding a supplement labeled as V-3FB. The enrichment in folic acid, cobalamine and biotin is based on their already proven beneficial effect on in vitro mammalian cell growth (Shane and Stockstand, 1985; Castro et al. 1992; Gebert and Gray, 1994). Benzoic acid supplement was considered for being in a much lesser amount than the other vitamins in BIOPRO1.

Table 2. Culture parameters of CHO cell cultures: Effect of vitamins supplementation.

		C1	C2
Parameter	CC	% control	
µ (h-1)	0.0160 ± 0.0015	113	114
Xv (106 cells ml-1)a	0.97 ± 0.04	111	113
CV (%)a	90.0 ± 2.0	104	102
qt-PA(ng 10-6 cells h-1)	24.5 ± 2.2	98	97
t-PA (mg l-1)a	1.50 ± 0.04	104	106
at-PA(U mg-1)	116.0 ± 8.4	96	97

a: 144 hrs of culture.

The results obtained in these experiments are presented in Figure 1 and Table 2. As shown in Figure 1a, supplement with V-TM allowed a maximum viable cell concentration (Xv) slightly but significantly higher than that obtained in CC. Similar results were obtained in C2 when V-3FB was supplemented, which is relevant in terms of cost. Specific growth rate (µ) in C1 and C2 was around 14% higher than in CC. These results indicate an increase both in cell concentration (stoichiometric improvement) and in specific cell growth rate (kinetic improvement), which is in agreement with results reported on other cell lines(Shane and Stockstand, 1985; Castro et al. 1992; Gebert and Gray, 1994). As seen in Figure 1b the increase in t-PA concentration correlates with the increase in Xv. On the other hand, despite its positive effect on cell growth, the increase in vitamin concentration did not affect the t-PA specific production rate (qtPA) nor its specific activity

(atPA), as shown in Table 2. The maximum levels of t-PA obtained in the different cultures (Table 2) were of the same order of magnitude of those reported for this cell line in the literature, that is, from 0.2 to 5 mg l-1 (Hansen and Emborg, 1994; Altamirano et al. 2001; Altamirano et al. 2004).

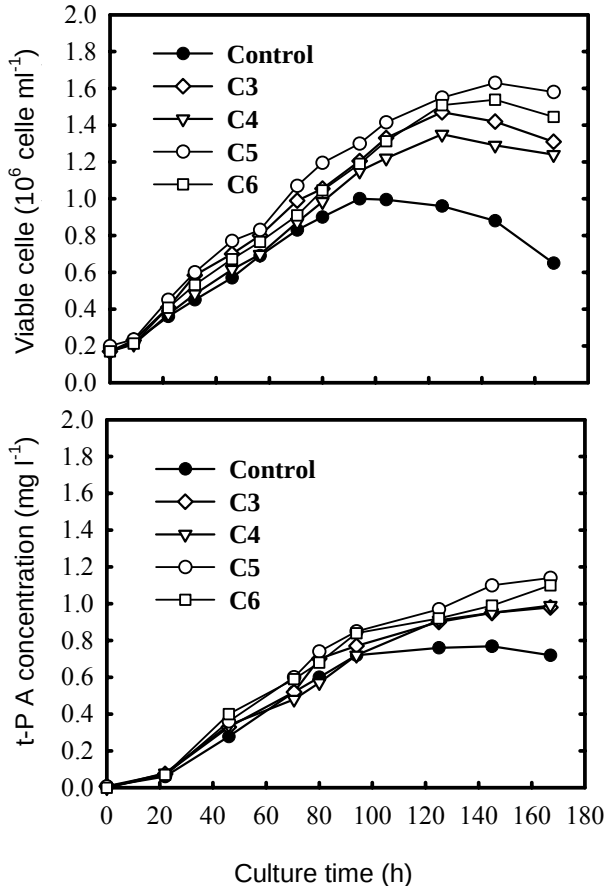

Figure 2. Effect of fatty acids and cholesterol supplementation on CHO cells growth (a) and t-PA concentration (b). Results of the batch cultures using BIOPRO 1 (CC), BIOPRO 1 plus 1X DLC and 3X V-TM (C3), BIOPRO 1 plus 1.5X DLC and 3X V-TM (C4), BIOPRO 1 plus 1X DLC and 6X V-TM (C5), and BIOPRO 1 plus 1.5X DLC and 6X V-TM (C6).

For the following series of cultures (S2), BIOPRO 1 supplemented with V-3FB was chosen to evaluate the enrichment with DLC since BIOPRO 1 has a limited spectrum of lipids. DLC was added to obtain a cholesterol concentration in the medium of 20 mg/ml and 30 mg/ml in C3 y C4 respectively (Table 1). In addition, at those two levels, vitamin supplement was further increased by doubling the amount of V-3FB added (C5 and C6) as shown in Table 1. Most relevant results of S2 are summarized in Figure 2 and Table 3. As seen in Figure 2a, the addition of DLC at both levels promoted a substantial increase in cell concentration (Xv) and cell viability (CV), best results being obtained in C4 which was the one with the highest content of lipids though not of vitamins. In this case a 65% increase in μ and 85% in Xv was obtained with respect to CC (Table 3) and growth phase was prolonged by 30 hrs. Doubling V-3FB (C6), instead produced a 10% decrease in maximum Xv with respect to C4, probably because some of the vitamins in V-3FB reached a level that is inhibitory or toxic to the cells. The beneficial effect t of lipids and cholesterol supplement on cell growth is related to the creation of a less aggressive environment for the culture, since it decreases shear stress, strengthen the plasmatic membrane and provides complex structural blocks that represent energy savings for the cell.

As seen in Figure 2b, higher t-PA concentration was obtained in the culture in which cell growth was higher (C4), with an increase of 31% with respect to CC (Table 3). In this case, the addition of lipids had opposite effects on t-PA production, increasing atPA while decreasing qtPA. A possible explanation is that cholesterol stiffens the plasmatic membrane, which increases its mechanical strength and therefore confers a higher viability to the cells (Jenkins et al. 1994; Castro et al. 1995). This implies a lesser protease release by cell lysis and increased product stability. However, this membrane stiffening may hinder the excretion of t-PA, which would explain the decrease in qtPA. This could be tested by measuring intracellular t-PA concentration. This aspect was not considered here but it is worthwhile studying in the future. Similar results have been obtained with γ-interferon-producing CHO K1 cells (Castro et al. 1995). Therefore no further increase in lipid supplementation was considered.

Table 3. Culture parameters of CHO cell cultures: Effect of fatty acids and cholesterol supplementation.

		C3	C4	C5	C6
Parameter	**CC**	**% control**			
μ (h^{-1})	0.0217 ± 0.0020	150	165	141	152
Xv (10^6 cel ml^{-1}) [a]	0.88 ± 0.05	161	185	146	175
CV (%)	88.0 ± 3.0	105	110	104	108
$q_{t\text{-}PA}$(ng 10^{-6} cells h^{-1})	19.80 ± 1.62	88	84	87	90
t-PA (mg l^{-1})[a]	0.80 ± 0.04	123	131	123	129
$a_{t\text{-}PA}$(U mg^{-1})[a]	116.0 ± 9.5	128	136	125	132

a: 145 hrs of culture.

To design the S3 experiments, the residual concentration of amino acids in C4 was analyzed. Apart from glutamine, the lower residual amino acid concentrations corresponded to serine, asparagine and proline (Figure 3). In the CC of S3 a similar concentration profile was obtained (data not shown) even though the specific consumption rate of serine and asparagine was higher. Serine and asparagine are not considered essential amino acids for CHO cells; however, high consumption rates have been determined in other CHO cell lines (Castro et al. 1995). Alanine, which usually accumulates during cultivation, competitively inhibits proline intake, therefore impairing CHO cell growth (Curriden and Englesberg, 1981). Alanine at 3 mM was reported inhibitory for growth of CHO-γ cells (Hayter et al. 1991). This might have occurred in C4, where proline

consumption rate decreased drastically over 2.4 mM alanine (data not shown).

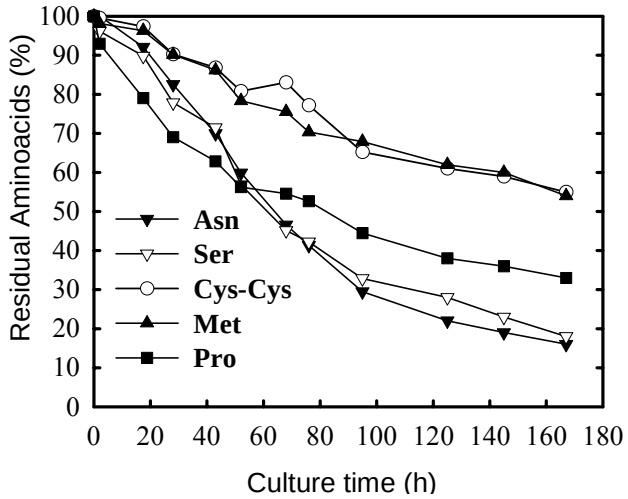

Figure 3. Amino acid consumption profile during batch culture of t-PA producing CHO cells on BIOPRO 1 plus 1.5X DLC and 3X V-TM (C4).

Therefore, in the S3 experiments the BIOPRO1 medium used in C4 was supplemented with 0.75 mmol of serine and 0.45 mmol of proline per litre of medium in C7 and with 0.75 mmol of serine, 0.45 mmol of proline and in addition 0.65 mmol of asparagine per liter of medium in C8, to assess the specific effect on asparagine. This effect is not apparent, since this amino acid could be limiting (it was consumed almost completely) while contributing to increase the level of ammonia. As seen in Figure 4 and Table 4, proline and serine addition (C7) allowed an increase in cell concentration over 130% with respect to CC, which represents an additional increase of 45% to that already obtained in C4. Increase of t-PA concentration was 42% and increase in specific activity was 36% with respect CC. Best results were obtained in C8, where cell concentration increased over threefold with respect to CC, while qtPA, reduced in C4 because of the addition of 30 µg ml-1 of DLC, was restored to a value close to that in CC. This allowed a 58% increase in t-PA concentration while maintaining a high level of specific activity (34% over CC). Although there is no clear explanation for this behaviour, which can only be attributed to the increase in asparagine concentration, stimulatory effect of specific amino acids on protein production has been reported in the case of arginine in the production of thrombopoietin with CHO cells (Chung et al. 2001). No effect on t-PA identity was observed in any case, as revealed by Western Blot (data not shown). Even though the medium was enriched in amino acids not considered essential for CHO cell growth, its supplement allowed the cells to take up these compounds from the medium instead of spending energy in its synthesis, which has a beneficial impact on cell growth. Besides, amino acids are not exclusively used for protein synthesis, but its degradation products converge to the TCA cycle furnishing energy or intermediary metabolites for the synthesis of other biomolecules like DNA and RNA.

As shown in Table 4, despite of the improvements obtained by medium supplementation with vitamins, lipids and amino acids, glucose and glutamine metabolism was barely affected. In fact, yields of glucose to lactate (YLac/Glc) and ammonia to glutamine (YAmm/Gln) were similar in all cultures. Besides, their specific consumption rates were very high, which is not surprising for in vitro mammalian cell cultures. YLac/Glc varies between 1.8 y 2.1 mol mol-1, while YAmm/Gln varied between 1.05 a 1.09 mol mol-1 revealing the highly inefficient use of these nutrients. YLac/Glc between 1.6 a 1.8 mol mol-1 and YAmm/Gln around 0.9 mol mol-1 have been reported for several cell lines (Xie and Wang, 1996; Cruz et al. 1999; Lee et al. 2003) grown on serum-based media, which can explain the difference with the values here reported. However, YLac/Glc for hybridoma cells grown on serum-free media are higher than those obtained in serum-based medium (Hiller et al. 1994), which is the same tendency observed in this case with CHO cells.

Table 4. Culture parameters of CHO cell cultures: Effect of specific amino acids supplementation.

		C7	C8
Parameter	CC	% control	
µ (h-1)	0.0180 ± 0.0019	159	162
Xv (106 cel ml-1)a	0.72 ± 0.04	230	251
CV (%)a	76.0 ± 4.0	130	139
qt-PA (ng 10-6 cells h-1)	21.8 ± 2.0	87	98
t-PA (mg l-1)a	1.01 ± 0.06	142	158
at-PA(U mg-1)a	102.0 ± 7.1	136	134
qGlc (nmol 10-6 cells h-1)	181.0 ± 5.4	95	94
qLac(nmol 10-6 cells h-1)	362 ± 14	89	92
qGln(nmol 10-6 cells h-1)	87.0 ± 4.5	83	80
qAmm(nmol 10-6 cells h-1)	130.0 ± 8.7	70	94

a: 146 hrs of culture

It is a well-known fact that in mammalian cell cultures glucose is converted to lactate at high yield (typically 1 to 2 mol/mol) and only a minor fraction of its carbon skeleton is metabolized through the TCA or the pentose phosphate cycle. A high rate of aerobic glycolysis has been explained in terms of glycolytic regulation disruption due to the non-glucose 6-phosphate regulated membrane-associated hexokinase activity and/or the low activity of pyruvate dehydrogenase and pyruvate carboxylase, which impairs a proper adjustment of glycolytic and TCA fluxes (Irani et al. 2002). Ammonia can be derived directly from the culture medium or else be a product of cell metabolism. In both cases glutamine is involved. Under culture conditions, glutamine suffers severe chemical decomposition to ammonia and pirrolidin carboxylate (Ozturk and Palsson, 1990). On the other hand, during catabolism (glutaminolysis) glutamine suffers double deamination, first to glutamate and then to α-ketoglutarate, which results in ammonia accumulation in the medium.

Fortification of BIOPRO1 medium with specific vitamins and amino acids, fatty acids and cholesterol did not preclude from high rates of glucose and glutamine consumption and the correspondingly high rates of undesirable byproducts formation. However it allowed increasing Xv significantly and prolonging the exponential growth phase in batch cultures of CHO TF 70R cell line. As a consequence a much higher concentration of t-PA (over 80%) and specific activity of t-PA (atPA, over 35%) were obtained, which is certainly a very relevant asset for t-PA production.

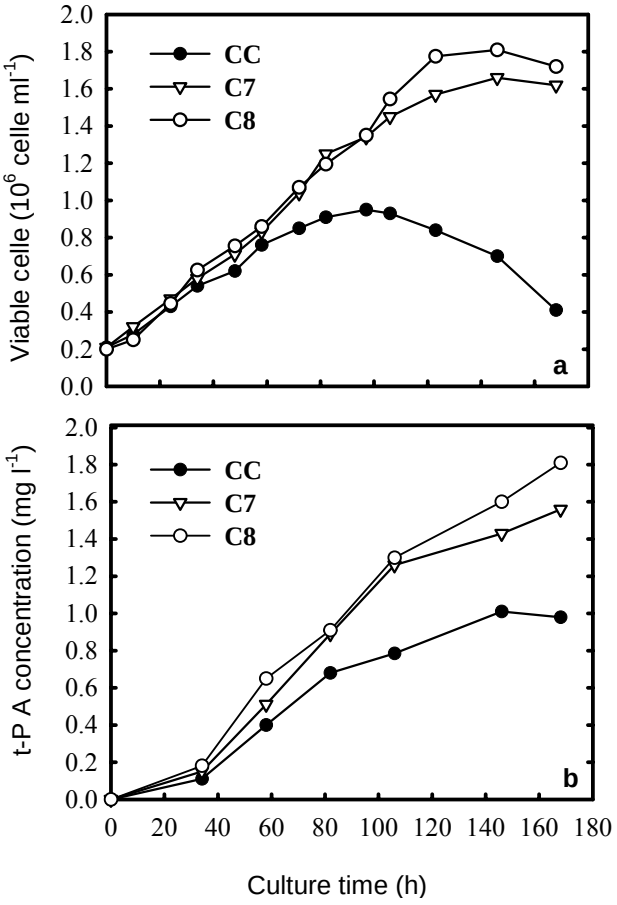

Figure 4. Effect of specific amino acid supplementation on CHO cells growth (a) and t-PA concentration (b). Results of the batch cultures using BIOPRO 1 (CC), BIOPRO 1 plus proline and serine, 1.5X DLC and 3X V-TM (C7) and BIOPRO 1 plus proline, serine, and asparagine, 1.5X DLC and 3X V-TM (C8).

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